Redirection of peroxisomal alcohol oxidase of Hansenula polymorpha to the secretory pathway

van der Heide, M; Leão, A.N; van der Klei, I.J.; Veenhuis, M

Published in:
Fems Yeast Research

DOI:
10.1111/j.1567-1364.2007.00225.x

Publication date:
2007

RESEARCH ARTICLE

Redirection of peroxisomal alcohol oxidase of *Hansenula polymorpha* to the secretory pathway

Meis van der Heide, Adriana N. Leão, Ida J. Van der Klei & Marten Veenhuis

Eukaryotic Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Haren, The Netherlands

Correspondence: M. Veenhuis, Eukaryotic Microbiology, University of Groningen, P.O. box 14, 9750 AA Haren, The Netherlands. Tel.: +31 50 3632176; fax: +31 50 3638280; e-mail: M.Veenhuis@rug.nl

Received 13 December 2006; revised 29 January 2007; accepted 30 January 2007. First published online 10 April 2007.

DOI:10.1111/j.1567-1364.2007.00225.x

Editor: Gerd Gellissen

Keywords
protein secretion; yeast; *Hansenula polymorpha*; alcohol oxidase; heterologous gene expression.

Abstract

We report on the rerouting of peroxisomal alcohol oxidase (AO) to the secretory pathway of *Hansenula polymorpha*. Using the leader sequence of the *Saccharomyces cerevisiae* mating factor α (MFα) as sorting signal, AO was correctly sorted to the endoplasmic reticulum (ER), which strongly proliferated in these cells. The MFα presequence, but not the prosequence, was cleaved from the protein. AO protein was present in the ER as monomers that lacked FAD, and hence was enzymatically inactive. Furthermore, the recombinant AO protein was subject to gradual degradation, possibly because the protein did not fold properly. However, when the *S. cerevisiae* invertase signal sequence (ISS) was used, secretion of AO protein was observed in conjunction with bulk of the protein being localized to the ER. The amount of secreted AO protein increased with increasing copy numbers of the AO expression cassette integrated into the genome. The secreted AO protein was correctly processed and displayed enzyme activity.

Introduction

The yeast *Hansenula polymorpha* is able to grow on methanol as the sole carbon and energy source. Growth on this compound is associated with induction of the synthesis of a specific set of proteins, which catalyze the conversion of methanol into biomass and energy (for a recent review, see Van der Klei *et al.*, 2006). Alcohol oxidase (AO) is the first enzyme of methanol metabolism, and catalyzes the oxidation of methanol to formaldehyde and hydrogen peroxide. The enzymatically active form of AO is a homo-octamer of *c.* 600 kDa. Each subunit contains one FAD molecule as the cofactor (Ozimek *et al.*, 2005). AO is a peroxisomal protein, and is sorted to peroxisomes in a Pex5p-dependent manner (Ozimek *et al.*, 2005).

During the past few years, methylotrophic yeast species, including *H. polymorpha*, have been recognized as attractive hosts for the synthesis and secretion of heterologous proteins (Gellissen & Melber, 1996; Gellissen *et al.*, 2005). Many proteins have been successfully produced on an industrial scale; a prominent example is a hepatitis B antigen that is produced in huge amounts in *H. polymorpha* for use in human healthcare (Hollenberg & Gellissen, 1997).

Oxidoreductases (such as AO) represent a class of proteins of strong scientific and industrial interest. In this study, we have used AO to investigate whether secretion of the enzymatically active enzyme via the secretory pathway could be achieved. The results are included in this article.

Materials and methods

Organisms and growth conditions

All *H. polymorpha* strains used are derivatives of NCYC495 (Gleeson & Sudbery, 1988). Wild-type (WT) strains (NCYC495 *leu1.1 ura3* and NCYC495 *leu1.1*), WT::P_{AOX}MFα*AOX*^{mc}, Δ*aox*::P_{AOX}MFα*AOX*, Δ*pex*3 (Baerends *et al.*, 1996), Δ*aox*::P_{AOX}MFα*AOX*/Δ*pex*3, WT::P_{AOX}ISS*AOX* with various copy numbers and Δ*aox*::P_{AOX}ISS*AOX* were grown at 37 °C in: (1) rich complex medium (YPD) containing 1% yeast extract, 2% peptone, and 1% glucose; (2) mineral medium (MM) as previously described (Van Dijken *et al.*, 1976); or (3) YNB without amino acids containing 0.67% Yeast Nitrogen Base (Difco). The carbon sources used were 0.5% glucose, 0.5% methanol or 0.1% glycerol/0.5% methanol; 0.25% ammonium sulfate was used as the nitrogen source 0.25%. Cultivation in continuous culture was performed at 37 °C in MM (Van Dijken *et al.*, 1976) supplemented with 0.2% glucose and 0.25% methanol at a dilution

rate of $0.1\,h^{-1}$. Leucine and/or uracil ($30\,\mu g\,mL^{-1}$) or riboflavin (1 mM) were added when required.

Escherichia coli DH5α (Gibco-Brl, Gaithesburg, MD) was grown at 37 °C in Luria–Bertani medium, supplemented with ampicillin ($100\,\mu g\,mL^{-1}$), kanamycin ($50\,\mu g\,mL^{-1}$) or erythromycin ($150\,\mu g\,mL^{-1}$) when required.

Miscellaneous DNA techniques

Standard recombinant DNA techniques, *E. coli* transformation and plasmid isolation procedures were performed essentially as previously described (Sambrook *et al.*, 1989). *Hansenula polymorpha* was transformed by electroporation (Faber *et al.*, 1994b).

Construction of plasmids containing endoplasmic reticulum (ER)-targeted *H. polymorpha* AO

The plasmid containing the *Saccharomyces cerevisiae* mating factor α (MFα) leader sequence fused in-frame to the *H. polymorpha AOX* gene under control of the AO promoter (P_{AOX}) was constructed as follows. A 1.8-kb fragment containing the P_{AOX} and MFα was isolated by PCR with the MFα primer (5′-GGG GGA TCC TCT TTT ATC CAA GCT TAC CCC) and the M13/pUC universal sequencing primer. The amplified fragment was digested with StuI and BamHI and cloned into pHIPX4-*PAS3* (Kiel *et al.*, 1995) digested with StuI and BamHI, resulting in plasmid pHIPX4-MFα-*PAS3*. The *AOX* gene was amplified using PCR and the AOX_{start} primer (5′-CCC GGA TCC GCC ATT CCT GAC GAA TTC G) and the AOX_{stop} primer (5′-CC CCC GGG TTA GAA TCT GGC AAG TCC GGT CTC C). Finally, the amplified *AOX* fragment was digested with BamHI and SmaI and cloned into BamHI/SmaI-digested pHIPX4-MFα-*PAS3*, thereby replacing the *PAS3* gene. The plasmid pHIPX4-MFα-*AOX* was used to transform NCYC495 *leu 1.1* cells. Correct integration into the P_{AOX} locus in multiple copies was confirmed by Southern blot analysis (data not shown).

The fragment used to replace the endogenous *AOX* gene was constructed as follows. A 4.1-kb StuI/NarI fragment from pHIPX4-MFα-*AOX* was cloned into pHIPX1 (Faber *et al.*, 1994a) digested with the same enzymes. The resulting plasmid was digested with StuI and NcoI, and the obtained 6.7-kb fragment was used to transform NCYC495 *leu1.1 ura3* cells. Correct integration into the P_{AOX} locus and replacement of the chromosomal *AOX* gene was confirmed by Southern blot analysis (data not shown). The resulting strain was designated AOΔ::P_{AOX}MFα*AOX*.

The double mutant AOΔ::P_{AOX}MFα*AOX*/*pex3* was obtained by crossing the AOΔ::P_{AOX}MFα*AOX* and *pex3* (Baerends *et al.*, 1996) mutants and selecting among the progeny for mutants containing both the AO and the *pex3* deletions.

The plasmid containing the *S. cerevisiae* invertase signal sequence (ISS) fused in-frame to the *H. polymorpha AOX* gene under control of the AO promoter (P_{AOX}) was constructed as follows. First, a plasmid containing the ISS fused to *Aspergillus niger* glucose oxidase was partially digested with NheI, and the isolated 8.9-kb fragment was then digested with SmaI, resulting in a 7.1-kb vector fragment. The *AOX* gene was amplified using PCR and the AOX_{start} primer (5′-CCC GGA TCC GCC ATT CCT GAC GAA TTC G) and the AOX_{stop} primer (5′-CC CCC GGG TTA GAA TCT GGC AAG TCC GGT CTC C). The amplified *AOX* fragment was digested with NheI and SmaI, and cloned into the NheI/SmaI-digested vector fragment. The plasmid pHIPX4-ISS*AOX* was used to transform NCYC495 *leu1.1* cells. Correct integration into the P_{AOX} locus was confirmed by Southern blot analysis (data not shown). Strains with various copies of the expression cassette integrated were selected for further analysis.

The fragment used to replace the endogenous *AOX* gene was constructed as follows. A 3.9-kb StuI/NarI fragment from pHIPX4-ISS*AOX* was cloned into pHIPX1 digested with the same enzymes. The resulting plasmid was digested with BamHI, and the 8.6-kb fragment obtained was used to transform NCYC 495 *leu1.1* cells. Correct integration into the P_{AOX} locus and replacement of the chromosomal *AOX* gene was confirmed by Southern blot analysis (data not shown). The resulting strain was designated AOΔ::P_{AOX}IS-S*AOX*.

Biochemical methods

Cell extracts were prepared as described previously (Van der Heide *et al.*, 2002). Separation of monomeric and octameric AO was performed by sucrose gradient centrifugation (Evers *et al.*, 1996). FAD was measured spectroscopically (Boteva *et al.*, 1999). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting and determination of AO activity followed established procedures. Protein concentrations were determined using the Biorad Protein Assay (Biorad GmbH, Munich, Germany), using bovine serum albumin as a standard.

Deglycosylation using endoglycosidase H (endo H) (Boehringer) was performed as follows. A volume of a yeast culture corresponding to three $OD_{660\,nm}$ units (volume × $OD_{660\,nm}$) was harvested by centrifugation. The cell pellet was resuspended in a 12.5% (w/v) trichloroacetic acid and frozen at −80 °C. After thawing, cells were collected by centrifugation at room temperature. The cell pellet was washed twice with ice-cold 80% acetone, and subsequently dissolved in 80 μL of 0.1 M NaOH/1% sodium dodecyl sulfate (SDS)/1% β-mercaptoethanol. After a clarifying spin, the cell extract was boiled for 5 min. For endo H treatment, 15 μL of the extract was added to 35 μL of endo H

buffer (80 mM sodium acetate, pH 4.5, 0.8% SDS, 1% β-mercaptoethanol) containing 2 mU of endo H (Boehringer) and protease inhibitors, and incubated for 2 h at 37 °C. Finally, SDS sample buffer was added, and the samples were boiled for 5 min prior to SDS-PAGE and Western blotting.

Inhibition of glycosylation by tunicamycin was obtained as follows. WT::P_{AOX}ISS*AOX*^{mc} cells were grown in methanol containing medium for 12 h. Then ($t = 0$ h), the culture was divided into two halves. To one half, tunicamycin (15 μg mL^{-1}) was added; the other half of the culture was used as a control. Samples, taken at $t = 0$ and 1 h, were analyzed by Western blotting using α-AO antibodies. The relative AO protein levels were determined by densitometry scanning of the blots.

Electron microscopy

Whole cells were fixed and embedded in Epon 812 or Unicryl as described previously (Waterham *et al.*, 1994). Ultrathin Unicryl sections were labeled using polyclonal antibodies against various proteins and goat anti-rabbit antibodies conjugated to gold, according to the instructions of the manufacturer (Amersham, UK). Cytochemical localization of AO activity was performed as previously described (Veenhuis *et al.*, 1976).

Results

Peroxisomal AO protein can enter the secretory pathway

In order to direct *H. polymorpha* AO into the secretory pathway, a hybrid gene was constructed encoding the leader sequence of the *S. cerevisiae* mating factor α (MFα) fused to *H. polymorpha* AO separated by a KEX2p cleavage site. This gene was placed under control of the inducible AO promoter (P_{AOX}), and integrated into the genome of WT *H. polymorpha*. Strains were selected that contained multiple copies (>3 copies) of the expression cassette in the genome, as confirmed by Southern blotting (data not shown).

The resulting strain, WT::P_{AOX}MFα*AOX*^{mc}, grew normally on glucose, glycerol and methanol at rates identical to that of the WT host strain (data not shown). As expected, AO protein was not produced during growth of cells on glucose (data not shown). However, in Western blots prepared from cell extracts of methanol-grown cells, decorated with α-AO antibodies, two AO protein bands were observed (Fig. 1; lane 3). The lower protein band corresponds to WT AO protein, whereas the higher band corresponds to AO protein, which is glycosylated (Fig. 1; compare lanes 3 and 4) and, based on its molecular weight after endo H treatment, still contained the proregion of the MFα leader sequence.

AO protein and activity were never detected in the culture fluid of these cultures. This could be related to the AO

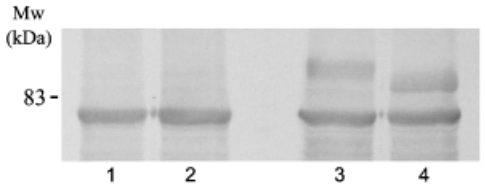

Fig. 1. Western blot prepared from samples after deglycosylation of cell extracts of methanol-grown NCYC495 *leu1.1* (WT) and WT::P_{AOX}MFα*AOX*^{mc} cells, decorated with α-AO antibodies. Lane 1: WT without endo H. Lane 2: WT with endo H. Lane 3: WT::P_{AOX}MFα*AOX*^{mc} without endo H. Lane 4: WT::P_{AOX}MFα*AOX*^{mc} with endo H. In extracts of WT::P_{AOX}MFα*AOX*^{mc} cells, two AO protein bands can be observed, representing WT peroxisomal AO (lower band) and, as judged from its molecular weight, MFαAO hybrid protein (lane 3). The deglycosylation data demonstrate that the MFαAO hybrid protein is glycosylated (compare lanes 3 and 4). Equal amounts of protein were loaded per lane.

production levels in cells grown in batch culture. Therefore, in order maximize the production levels of recombinant AO protein, strain WT::P_{AOX}MFα*AOX*^{mc} was grown in a carbon-limited chemostat culture ($D = 0.1$ h^{-1}) on a mixture of glucose and methanol. Electron microscopy revealed that cells grown under these conditions contained the expected numbers of peroxisomes (Van Dijken *et al.*, 1976), a phenomenon paralleled by strong proliferation of ER-like vesicles (Fig. 2a). In immunocytochemical experiments using α-AO antibodies, labeling was observed on both the peroxisomal profiles and these vesicles (Fig. 2b). Subsequent cytochemical experiments unequivocally showed that AO enzymatic activity was strictly confined to the peroxisomal matrix (Fig. 2c). These results suggested that the MFαAO protein that accumulated in the ER-like vesicles had no enzymatic activity and therefore was most likely present as monomers. To seek further evidence for this, we aimed to determine the oligomeric state of the MFαAO protein as well as the FAD content. To avoid possible interference by endogenous WT AO with the recombinant AO in the purification procedures, we decided to use a strain in which the endogenous *AOX* gene was replaced by the MFα*AOX* hybrid gene.

Analysis of an *H. polymorpha* AO-deficient strain producing MFαAO fusion protein

The *H. polymorpha* AOΔ::P_{AOX}MFα*AOX* strain was not able to grow on methanol (Mut$^-$ phenotype; data not shown). In *H. polymorpha* Mut$^-$ cells, however, high P_{AOX} induction levels can readily be achieved upon growth of cells in batch cultures on glycerol/methanol mixtures. In crude extracts, prepared from glycerol/methanol-grown AOΔ::P_{AOX}MFα*AOX* cells, AO activity was not detectable; enzymatic activity was also not observed in the culture fluid of these cultures. Morphologically, strong proliferation of vesicles was observed. These structures represented the sole site of

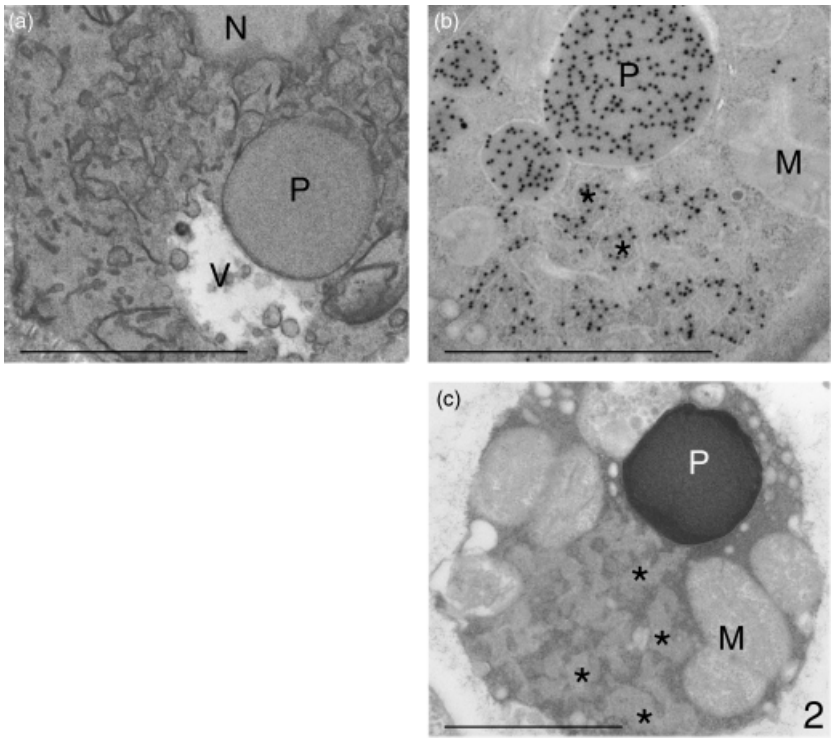

Fig. 2. Overall morphology (a) of methanol-grown WT::P_{AOX}MFα*AOX*^{mc} cells, showing the development of numerous vesicles. (b) Results of an immunocytochemical experiment using specific antibodies against AO. Labeling is located on peroxisomes, the nuclear membrane, and the vesicles, suggesting that the vesicles are of an ER nature. Immunocytochemically, AO activity is confined to peroxisomes (c). The proliferated ER vesicles (asterisks) show no reaction products, indicating the absence of AO activity. Protoplasts were incubated with $CeCl_3$ and methanol. M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. Bar = 0.5 μm.

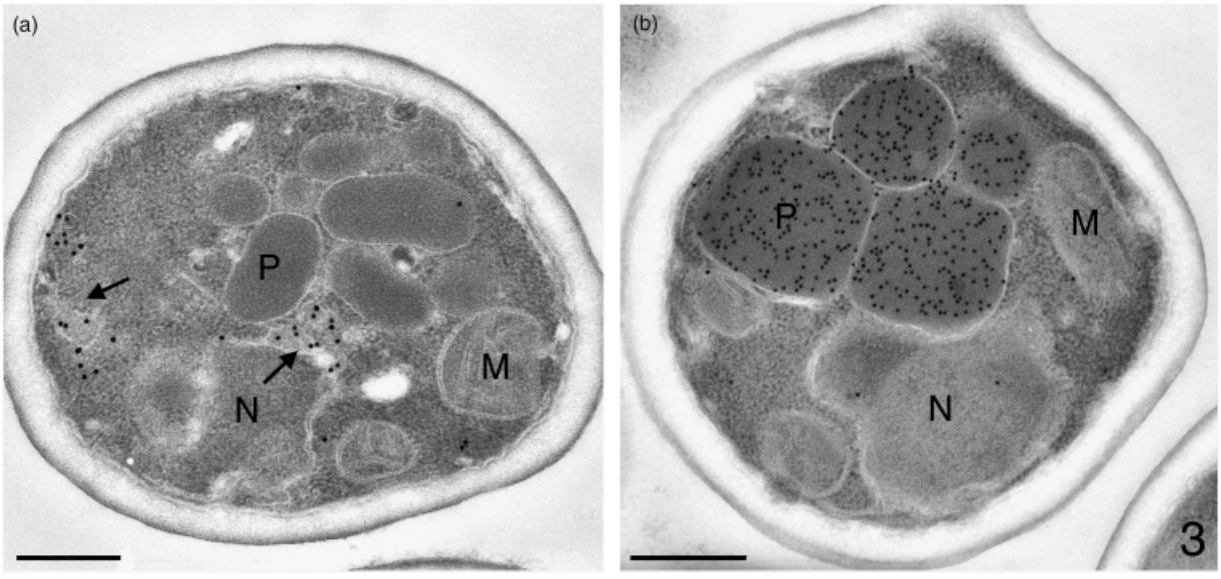

Fig. 3. Immunocytochemical experiments, using α-AO antibodies on ultrathin sections of Δ*aox*::P_{AOX}MFα*AOX* cells (a), grown on glycerol/methanol, showing the exclusive presence of AO protein in the vesicles. (b) A typical example of an identically grown WT *Hansenula polymorpha* cell in which AO protein is confined to peroxisomes. M, mitochondrion; N, nucleus; P, peroxisome. Arrows indicate ER-derived vesicles. Bar = 0.5 μm.

AO protein in the cells, as was evident from immunocytochemistry (Fig. 3a).

These experiments also revealed that peroxisome biogenesis was not severely affected, except that the organelles present in the AOΔ::P_{AOX}MFα*AOX* cells were smaller than WT peroxisomes. The latter can be readily explained by the fact that a major protein constituent, AO protein, was absent from these organelles but had accumulated in the ER. These data also imply that import of hybrid AO into peroxisomes did not occur. Apparently, the artificial N-terminal secretion signal overrules the authentic peroxisomal sorting machinery.

Characterization of MFαAO protein from AOΔ::P_{AOX}MFα*AOX* cells

Crude extracts, prepared from AOΔ::P_{AOX}MFα*AOX* and WT cells incubated in methanol media, were subjected to Western blot analysis using α-AO antibodies. These experiments revealed that the amount of AO protein present in AOΔ::P_{AOX}MFα*AOX* cells was reduced relative to the WT controls (Fig. 4).

To determine the oligomerization state and the FAD content of the vesicle-resident AO protein, crude extracts of glycerol/methanol-grown cells were subjected to sucrose gradient centrifugation. These data showed that AO from AOΔ::P_{AOX}MFα*AOX* cells was solely present at low density in the gradient, consistent with AO monomers (Fig. 5). The FAD content of these AO monomers was analyzed by fluorescence spectroscopy, which failed to resolve the presence of FAD. Taken together, these data indicate that the hybrid AO protein that had accumulated in the ER in AOΔ::P_{AOX}MFα*AOX* cells was exclusively present as monomers that had not bound FAD.

The finding that in AOΔ::P_{AOX}MFα*AOX* cells the AO levels are reduced compared to WT controls may be interpreted as meaning that the hybrid protein is less stable than its WT counterpart. To investigate this aspect in more detail, AOΔ::P_{AOX}MFα*AOX* cells were grown for 24 h in batch cultures on glycerol/methanol. Subsequently, excess glucose was added to the cultures, thus fully repressing the synthesis of AO protein. In WT cells, the addition of excess glucose leads to rapid degradation of the peroxisomes present in the cells via macropexophagy, and thus to a rapid decrease in the level of AO protein (Dunn *et al*., 2005; Sakai *et al*., 2006). The fate of AO protein present in the AOΔ::P_{AOX}MFα*AOX* cells was followed by Western blot analysis. The data presented in Fig. 6 show that the levels of AO protein gradually decreased. As this protein is not subject to macropexophagy, this suggests that the vesicle-resident AO is gradually degraded, possibly by the ER-associated degradation machinery (ERAD) (Werner *et al*., 1996). The reason for this degradation could be related to the fact that the ER

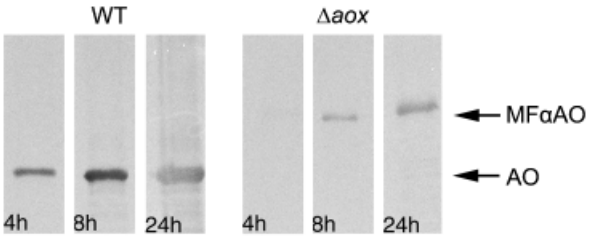

Fig. 4. Western blot analysis using α-AO antibodies and crude extracts of both WT and Δ*aox*::P_{AOX} MFα*AOX* cells, showing the induction of AO protein in these cells in batch cultures 4, 8 and 24 h after the shift of cells from glucose to methanol media. WT, wild type; Δ*aox*, Δ*aox*::P_{AOX} MFα*AOX*. Equal amounts of protein were loaded per lane.

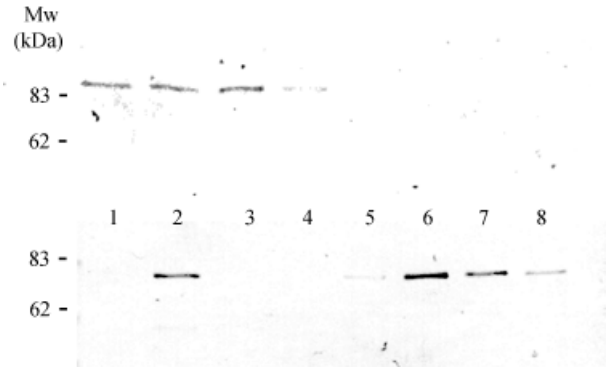

Fig. 5. Western blots, using α-AO antibodies, prepared from the various fractions obtained after sucrose gradient centrifugation of equal amounts of cell extracts of Δ*aox*::P_{AOX} MFα*AOX* cells (upper part) and WT *Hansenula polymorpha* (lower part), showing the exclusive presence of monomeric AO in the Δ*aox*::P_{AOX} MFα*AOX* strain. Lane 1 represents the top fraction of the gradient, and lane 8 the bottom fraction. Lanes 1–3: monomeric AO (72 kDa). Lanes 6–8: octameric AO (600 kDa). Mw, molecular mass in kDa.

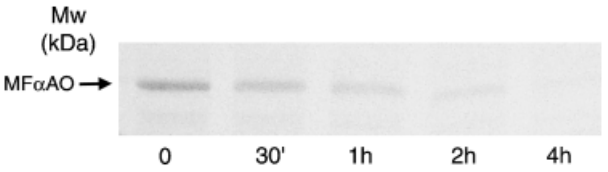

Fig. 6. Western blot analysis using crude extracts prepared from cells of Δ*aox*::P_{AOX}MFα*AOX* grown for 24 h on glycerol/methanol ($t = 0$ h) and subsequently exposed to 0.5% glucose for 30 min, 1, 2 and 4 h. The blot was decorated using α-AO antibodies. To correct for growth of the cells, equal culture volumes were loaded per lane. Reduction of the amount of AO protein over time is evident.

quality control system recognizes the hybrid AO protein as being improperly folded, a known phenomenon for AO under conditions in which binding of FAD is prevented (Boteva *et al*., 1999).

Probably, the MFα signal sequence results in post-translational import of the hybrid AO protein into the ER (Brodsky *et al*., 1995), and thus the protein is expected to be completely synthesized in the cytosol prior to import into this compartment. This property allowed to investigate whether the hybrid AO protein was intrinsically competent to assemble into an active enzyme. This assumption is based on earlier observations that putative peroxisomal AO assembly factors are also functional in the cytosol, as exemplified by the presence of active oligomeric AO in the cytosol of peroxisome-deficient (*pex*) mutant cells that lack intact peroxisomes (Ozimek *et al*., 2005).

Therefore, we studied the fate of MFαAO in a *pex3*/AOΔ::P_{AOX}MFα*AOX* double mutant. As expected, methanol-induced cells of this strain lacked peroxisomes (data not shown). In addition, in crude extracts prepared from such

cells, AO activity was invariably undetectable (data not shown). These results suggest that putative peroxisomal AO assembly factors, which are functional in the cytosol of *pex* mutants, were not able to perform their function in the cytosol of the double mutant cells.

In earlier studies, we demonstrated that the availability of FAD is of crucial importance for the correct assembly of AO (Evers *et al*., 1994, 1996). To investigate the possibility that limiting amounts of FAD could account for the default in MFαAO assembly, both the AOΔ::P_{AOX}MFα*AOX* strain and the *pex3*/AOΔ::P_{AOX}MFα*AOX* double mutant strain were grown on glycerol/methanol in the presence of 1 mM riboflavin. However, also under these conditions, AO activity could not be detected, consistent with the view that FAD is not the limiting factor in the AO assembly process in these cells (data not shown).

Analysis of strains producing an ISS–AO fusion protein

The results obtained indicated that the signal sequence of *S. cerevisiae* MFα efficiently sorted peroxisomal AO to the secretory pathway, although secretion was prohibited. We speculate that the inability to bind FAD was due to steric hindrance of the FAD-binding fold by the proregion of the leader sequence, as the FAD-binding site is near the N-terminus of the protein (Boteva *et al*., 1999). To test this possibility, we used an alternative sorting signal, namely the *S. cerevisiae* ISS. This signal differs from that of the MFα in that it consists only of a presequence, which is cleaved off upon import of the protein into the ER. As a result, the N-terminus of AO may be normally exposed under these conditions.

To obtain strains in which AO was sorted to the ER using the ISS, a hybrid gene was constructed encoding the ISS fused in-frame to the *H. polymorpha* AO gene. This gene was placed under the control of the inducible *AOX* promoter (P_{AOX}) and integrated at various copy numbers into the genome of WT *H. polymorpha*. The resulting strains grew on glucose at rates identical to the WT host strain (data not shown). In Western blots, prepared from crude extracts of methanol-grown cells of strains with different copy numbers, decorated with α-AO antibodies, two AO protein bands were observed (Fig. 7a). The lower band corresponds to normal WT AO protein. The higher band most likely represents glycosylated AO protein, as judged from a deglycosylation experiment, using endo H of strain WT::P_{AOX}ISS*AOX*^{mc} (Fig. 7b). Remarkably, AO protein was also detected in the culture fluid of the various constructed strains (Fig. 7c). From Fig. 7, it is evident that AO protein levels in the culture fluid increased with increasing copy number (Fig. 7c).

On the basis of its electrophoretic behavior, the AO protein detected in the culture fluid was of WT molecular

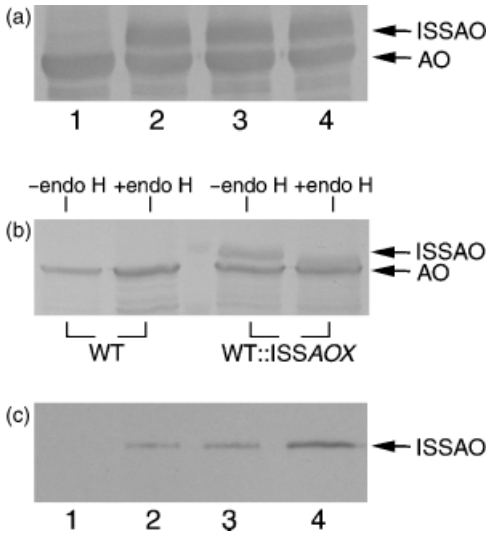

Fig. 7. (a) Western blot prepared from crude extracts of WT cells (lane 1), WT::P_{AOX}ISS*AOX*^{1c} cells (lane 2), WT::P_{AOX}ISS*AOX*^{2c} cells (lane 3), and WT::P_{AOX}ISS*AOX*^{mc} cells (lane 4). (b) Western blot prepared from crude extracts treated with endo H (+endo H) or without endo H (−endoH) used as controls. Lanes 1 and 2: extracts of WT cells treated with endo H (lane 2) or incubated without endo H (lane 1). Lanes 3 and 4: extracts of WT::P_{AOX}ISS*AOX*^{mc} without endo H (lane 3) or with endo H (lane 4). (c) Western blot prepared from the culture fluids of methanol-grown cells of WT (lane 1), WT:: P_{AOX}ISS*AOX*^{1c} (lane 2), WT::P_{AOX}ISS*AOX*^{2c} (lane 3), and WT::P_{AOX}ISS*AOX*^{mc} (lane 4). In crude extracts of the recombinant strains, two AO protein bands were observed, representing WT AO (lower band) and the recombinant AO protein (ISS–AO), which, on the basis of the endo H treatment, is glycosylated (b). Extracellular AO protein was only detected in supernatants of cultures of the recombinant strains (c). For crude extracts, equal amounts of protein were loaded per lane. For the cultivation media, a volume corresponding to 3 $OD_{660\,nm}$ units of cells was loaded per lane. Blots were decorated using α-AO antibodies. Cells were grown on methanol for 18 h.

mass and not glycosylated. Activity measurements revealed that the extracellular protein displayed enzymatic activity (data not shown). In addition, the increase in AO protein was paralleled by an increase in extracellular AO activity up to 4 mU mL^{-1} culture fluid.

To investigate the possibility that the extracellular AO is due to cell lysis, the WT::P_{AOX}ISS*AOX*^{mc} strain was grown on methanol, and the appearance of AO activity in the culture fluid was analyzed. This revealed that extracellular AO activity appears already during the mid-exponential growth phase, which makes lysis as the cause of extracellular AO highly unlikely (data not shown). When vital staining of cells from the exponential cultures was performed, dead cells were not observed (data not shown).

Other evidence arguing against possible cell lysis was that two other major components of the peroxisomal matrix, dihydroxy acetone synthase (DHAS) and catalase (CAT), and the cytosolic protein elongation factor 1α (EF1α), were

clearly present in cell extracts but not detectable in the culture fluid by Western blot analysis (data not shown). Moreover, enzyme measurement revealed that the cytosolic enzyme formate dehydrogenase (FDH) was readily detectable in cell extracts but absent in the culture fluid (data not shown).

To ensure that the extracellular AO was indeed originating from the ISS–AO fusion, the secreted AO protein was N-terminally sequenced. Here, we made use of the fact that removal of the ISS upon import of the recombinant AO into the ER would result in a serine residue as the first amino acid. Sequencing of the extracellular AO indeed revealed that the first amino acid residue was a serine, thus confirming the recombinant origin of the extracellular AO protein.

The amount of AO protein detected in the culture fluid was limited relative to the total amount of AO produced (compare Figs. 7a and c). This suggests that bulk of the AO protein had accumulated intracellularly. Indeed, immunocytochemistry, using α-AO antibodies, on WT::P_{AOX}ISS*AOX*^{mc} cells revealed that AO protein was present not only on the peroxisomal profiles but also on strongly proliferated ER-like structures (Figs. 8a and b). DHAS protein, however, was only present in peroxisomes, indicating that correct sorting of DHAS was unaffected in such cells (Fig. 8c).

To determine whether the hybrid AO protein was able to bind FAD, the recombinant protein was purified from an AOΔstrain producing the ISS–AO fusion protein. Immunocytochemistry revealed that, as in WT::P_{AOX}ISS*AOX* cells, strong proliferation of ER-like structures had occurred (Fig. 8d). These structures represented the sole sites of AO protein in the cells. Fluorescence analysis of the FAD content of recombinant AO, immunoprecipitated from crude extracts of AOΔ::P_{AOX}MFα*AOX*, revealed that FAD was present in such precipitates, although at relatively low amounts (data not shown). From this, we concluded that a significant proportion of the ISS–AO fusion protein had bound FAD.

As already indicated, cells of strain WT::P_{AOX}ISS*AOX*^{mc} are able to secrete active AO protein. However, the bulk of the recombinant protein remained intracellularly in a glycosylated form. Analysis of the AO protein sequence revealed that AO contains two putative *N*-glycosylation sites (Asn32

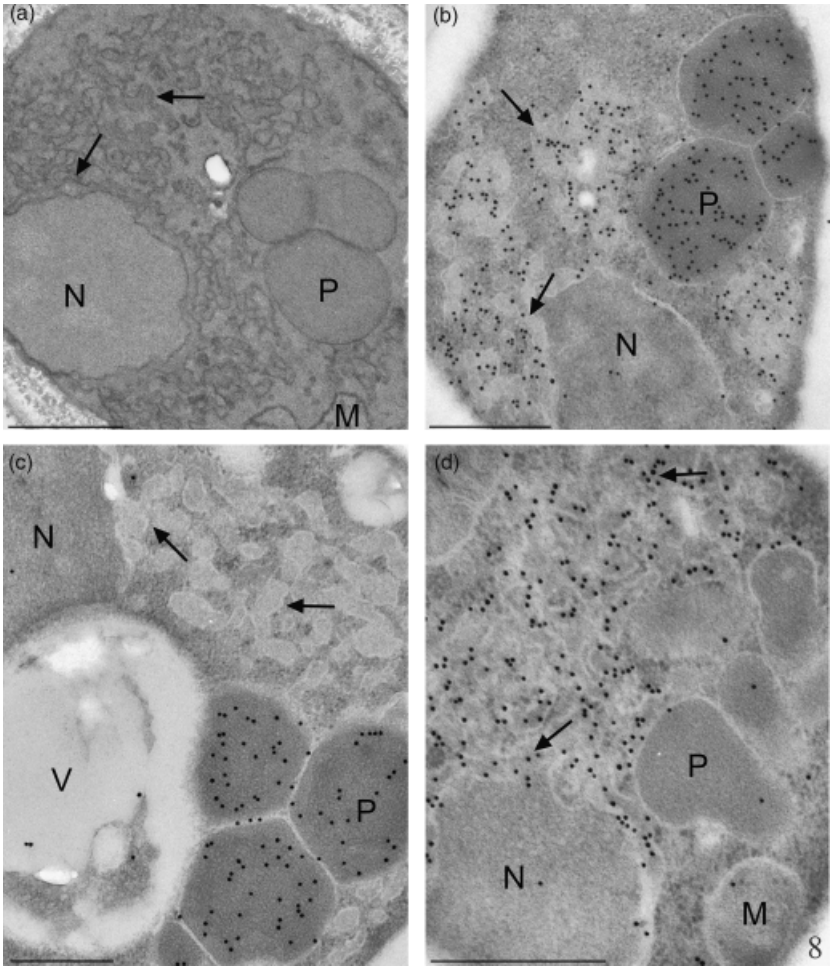

Fig. 8. Overall morphology (a) of methanol-grown WT::P_{AOX}ISS*AOX*^{mc} cells, showing the presence of normal peroxisomes and the strongly proliferated ER-like structures. (b, c) Immunocytochemical experiments using α-AO (b) or α-DHAS (c) antibodies. AO labeling is located on peroxisomes and the ER-like structures. DHAS labeling was exclusively located on peroxisomal profiles, indicating that DHAS sorting was unaffected in these cells. (d) After labeling of Δ*aox*::P_{AOX}ISS*AOX* cells using α-AO antibodies, AO protein is exclusively present on proliferated ER-like structures, but not on peroxisomes. N, nucleus; M, mitochondrion; P, peroxisome; V, vacuole. Arrows indicate ER-like structures. Bar = 0.5 μm.

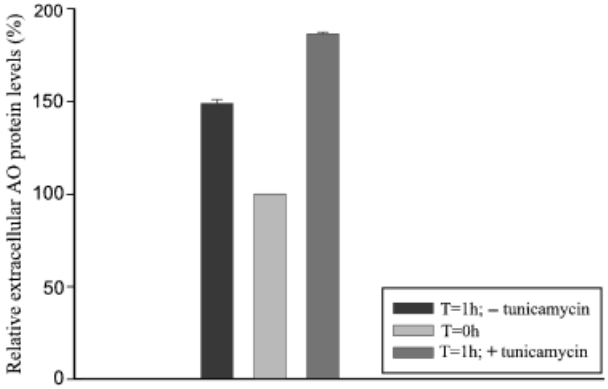

Fig. 9. Relative levels of extracellular AO protein in the presence or absence of tunicamycin as determined by scanning of Western blots, prepared from equal samples of the growth media. WT::P_{AOX}ISS*AOX*^{mc} cells were grown in methanol-containing medium for 12 h. At this time point ($t = 0$ h), tunicamycin was added, and cultures were incubated for 1 h. Addition of tunicamycin enhanced the level of extracellular AO protein. The values presented are the mean of two independent experiments. The amount of AO protein present at $t = 0$ h was arbitrarily set to 100%.

and Asn155). On the basis of molecular weight estimation of the produced AO protein, we propose that one of these sites is glycosylated *in vivo*. We hypothesized that glycosylation of the recombinant AO may lead to a nonexportable protein conformation. This would explain the observation that the extracellular AO, which is active, is not glycosylated. To investigate this possibility, we investigated the effect of tunicamycin, which is a known inhibitor of glycosylation, on the secretion levels of AO protein. The *H. polymorpha* WT::P_{AOX}ISS*AOX*^{mc} strain was grown on methanol for 12 h, when secretion of active AO had just started. Thereafter, tunicamycin was added to the culture. The results, shown in Fig. 9, indeed demonstrate that incubation of cells in the presence of tunicamycin leads to enhanced levels of extracellular AO protein.

Discussion

We have examined the fate of *H. polymorpha* AO upon artificial sorting of this peroxisomal matrix protein into the secretory pathway. WT *H. polymorpha* AO is an oligomeric flavoprotein that consists of eight identical subunits, each of which carries a flavin moiety (FAD) as the prosthetic group. AO is a typical example of a complex enzyme molecule that cannot spontaneously assemble *in vitro* from its constituent parts (recently reviewed by Ozimek *et al.*, 2005), and was used here as a model protein with which to investigate the requirements for the production and secretion of complex flavoproteins.

In methanol-grown *H. polymorpha*, AO is normally located in peroxisomes. To direct AO into the secretory pathway, we used the coding region of the leader sequence of *S. cerevisiae* MFα placed in front of the full-length homologous *H. polymorpha AOX* gene. The rationale of this approach was two-fold. First, the MFα signal functions well in *H. polymorpha*, as exemplified by the successful secretion of *A. niger* glucose oxidase (GOX), using this signal (Van der Heide *et al.*, 2002). It is relevant here that *A. niger* GOX is a dimeric flavoprotein that resembles AO in structure (Boteva *et al.*, 1999). Second, using the MFα leader sequence, the hybrid AO protein will most probably be post-translationally imported into the ER. This implies that the protein is completely synthesized in the cytosol prior to import into the ER, an order of events that largely resembles the normal import pathway of AO into peroxisomes.

Our data indicated that the MFαAO fusion protein is indeed sorted into the secretory pathway. Our results demonstrate that the hybrid AO protein remained in the secretory pathway and was not exported from the cell. Possibly, the conformation of the protein did not allow export. This hypothesis is consistent with the observation that the MFαAO fusion protein was exclusively present as a monomer that lacked FAD. The failure to bind FAD was not due to FAD limitation, as was evident from experiments performed under conditions of excess riboflavin. Monomeric AO, lacking FAD, is also highly unstable *in vitro* (De Hoop *et al.*, 1991), and readily aggregates, both *in vitro* and in intact cells. Thus, conformational changes of AO monomers related to the absence of FAD may cause the failure in secretion. Subsequently, the ER quality control system may recognize these proteins as being incorrectly folded, leading to the removal of these molecules by the ERAD machinery. This would be the most likely explanation for the observed gradual decrease of hybrid AO protein upon a shift of methanol-induced cells to conditions in which the AO protein is no longer synthesized.

The reason why MFαAO protein is not assembled is still not known with certainty. A likely explanation is based on the notion that the protein MFα presequence is not cleaved off, as suggested by the size of the protein relative to WT AO. The presence of this sequence could interfere (e.g. by steric hindrance or by affecting the structure) with the availability of the FAD-binding site, which is located at the N-terminus of the protein, and thus prevent binding of the cofactor. This would also be a plausible explanation for the finding that all MFαAO protein is directed to the ER. Gunkel *et al.* (2004) showed that Pex5p only binds AO when FAD is present. This possibility was strengthened by the data obtained using an alternative signal sequence to direct AO into the secretion pathway, namely the leader sequence of *S. cerevisiae* ISS. This signal differs from the MFα signal in that is spliced off during import of the protein into the ER, with the advantage that the N-terminus of the ER-resident AO is now unaltered and normally exposed at these conditions.

ISS–AO was efficiently targeted to ER-like structures, despite the fact that the peroxisomal targeting signal (ARF) is still present at the C-terminus of the protein. This location of ISS–AO was accompanied by strong proliferation of these ER-like structures. Peroxisome biogenesis, however, was almost unaffected under these conditions.

In addition, active AO protein could be detected in the culture fluid of methanol-grown WT::P_{AOX}ISS*AOX* cells. The presence of extracellular AO was not due to cell lysis, as demonstrated by the fact that neither DHAS nor CAT was detectable in the growth medium. Furthermore, the activities of both EF1α and formate dehydrogenase (FDH), both cytosolic proteins, were also invariably absent in the culture fluid under these conditions. The extracellular AO protein is of recombinant origin, as became clear from N-terminal sequence analysis. Probably, AO secretion can be increased using an engineered AOX gene in which possible glycosylation sites are mutated.

In summary, we have shown that it is possible to redirect a complex peroxisomal flavoprotein, namely AO, to the secretion machinery and allow secretion of enzymatically active protein. Apparently, the secretory signal is able to overrule the peroxisomal targeting signal. This opens the way to develop methylotrophic yeasts as efficient cell factories for the production of this industrially important group of enzymes.

Acknowledgements

We thank Klaas Sjollema and Ineke Keizer for technical assistance in electron microscopy. M. van der Heide is supported by STW, I.J. van der Klei by an ALW/NWO Pionier-grant, and A.N. Leão by a grant from the State University of Rio de Janeiro (Brazil).

References

Baerends RJS, Rasmussen SW, Hilbrands RE, van der Heide M, Faber KN, Reuvekamp PT, Kiel JAKW, Cregg JM, van der Klei IJ & Veenhuis M (1996) The *Hansenula polymorpha PER9* gene encodes a peroxisomal membrane protein essential for peroxisome assembly and integrity. *J Biol Chem* **271**: 8887–8894.

Boteva R, Visser AJ, Filippi B, Vriend G, Veenhuis M & Van der Klei IJ (1999) Conformational transitions accompanying oligomerization of yeast alcohol oxidase, a peroxisomal flavoenzyme. *Biochemistry* **38**: 5034–5044.

Brodsky JL, Goeckeler J & Schekman R (1995) BiP and Sec63p are required for both co- and posttranslational protein translocation into the yeast endoplasmic reticulum. *Proc Natl Acad Sci USA* **92**: 9643–9646.

De Hoop M, Asgeirsdottir S, Blaauw M, Veenhuis M, Cregg J, Gleeson M & AB G (1991) Mutations in the FAD-binding fold of alcohol oxidase from *Hansenula polymorpha*. *Protein Eng* **4**: 821–829.

Dunn WA, Cregg JM, Kiel JA, Van der Klei IJ, Oku M, Sakai Y, Sibirny AA, Stasyk OV & Veenhuis M (2005) Pexophagy: the selective autophagy of peroxisomes. *Autophagy* **1**: 75–83.

Evers ME, Titorenko VI, van der Klei IJ, Harder W & Veenhuis M (1994) Assembly of alcohol oxidase in peroxisomes of the yeast *Hansenula polymorpha* requires the cofactor flavin adenine dinucleotide. *Mol Biol Cell* **5**: 829–837.

Evers ME, Titorenko V, Harder W, Van der Klei IJ & Veenhuis M (1996) Flavin adenine dinucleotide binding is the crucial step in alcohol oxidase assembly in the yeast *Hansenula polymorpha*. *Yeast* **12**: 917–923.

Faber KN, Haima P, Gietl C, Harder W, AB G & Veenhuis M (1994a) The methylotrophic yeast *Hansenula polymorpha* contains an inducible import pathway for peroxisomal matrix proteins with an N-terminal targeting signal (PTS2 proteins). *Proc Natl Acad Sci USA* **91**: 12985–12989.

Faber KN, Haima P, Harder W, Veenhuis M & AB G (1994b) Highly-efficient electrotransformation of the yeast *Hansenula polymorpha*. *Curr Genet* **25**: 305–310.

Gellissen G & Melber K (1996) Methylotrophic yeast *Hansenula polymorpha* as production organism for recombinant pharmaceuticals. *Arzneimittelforschung* **46**: 943–948.

Gellissen G, Kunze G, Gaillardin C, Cregg JM, Berardi E, Veenhuis M & Van der Klei I (2005) New yeast expression platforms based on methylotrophic *Hansenula polymorpha* and *Pichia pastoris* and on dimorphic *Arxula adeninivorans* and *Yarrowia lipolytica*: a comparison. *FEMS Yeast Res* **5**: 1079–1096.

Gleeson MAG & Sudbery PE (1988) Genetic analysis in the methylotrophic yeast *Hansenula polymorpha*. *Yeast* **4**: 293–303.

Gunkel K, van Dijk R, Veenhuis M & van der Klei IJ (2004) Routing of *Hansenula polymorpha* alcohol oxidase: an alternative peroxisomal protein-sorting machinery. *Mol Biol Cell* **15**: 1347–1355.

Hollenberg CP & Gellissen G (1997) Production of recombinant proteins by methylotrophic yeasts. *Curr Opin Biotechnol* **8**: 554–560.

Kiel JAKW, Keizer-Gunnink IK, Krause T, Komori M & Veenhuis M (1995) Heterologous complementation of peroxisome function in yeast: the *Saccharomyces cerevisiae PAS3* gene restores peroxisome biogenesis in a *Hansenula polymorpha per9* disruption mutant. *FEBS Lett* **377**: 434–438.

Ozimek P, Veenhuis M & Van der Klei IJ (2005) Alcohol oxidase: a complex peroxisomal, oligomeric flavoprotein. *FEMS Yeast Res* **5**: 975–983.

Sakai Y, Oku M, Van der Klei IJ & Kiel JA (2006) Pexophagy: autophagic degradation of peroxisomes. *Biochim Biophys Acta* **1763**: 1767–1775.

Sambrook J, Fritsch EF & Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*. Cold Sping Harbor Laboratory Press, Cold Spring Harbor, NY.

Van der Heide M, Hollenberg CP, Van der Klei IJ & Veenhuis M (2002) Overproduction of BiP negatively affects the secretion of *Aspergillus niger* glucose oxidase by the yeast *Hansenula polymorpha*. *Appl Microbiol Biotechnol* **58**: 487–494.

Van der Klei IJ, Yurimoto H, Sakai Y & Veenhuis M (2006) The significance of peroxisomes in methanol metabolism in methylotrophic yeast. *Biochim Biophys Acta* **1763**: 1453–1462.

Van Dijken JP, Otto R & Harder W (1976) Growth of *Hansenula polymorpha* in a methanol-limited chemostat. Physiological responses due to the involvement of methanol oxidase as a key enzyme in methanol metabolism. *Arch Microbiol* **111**: 137–144.

Veenhuis M, Van Dijken JP & Harder W (1976) Cytochemical studies on the localization of methanol oxidase and other oxidases in peroxisomes of methanol-grown *Hansenula polymorpha*. *Arch Microbiol* **111**: 123–135.

Waterham HR, Titorenko VI, Haima P, Cregg JM, Harder W & Veenhuis M (1994) The *Hansenula polymorpha PER1* gene is essential for peroxisome biogenesis and encodes a peroxisomal matrix protein with both carboxy- and amino-terminal targeting signals. *J Cell Biol* **127**: 737–749.

Werner ED, Brodsky JL & McCracken AA (1996) Proteasome-dependent endoplasmic reticulum-associated protein degradation: an unconventional route to a familiar fate. *Proc Natl Acad Sci USA* **93**: 13797–13801.